Original Research Article

Effect of Grape Seed Oil on Chronic Carbon Tetrachloride-Induced Hepatic Injury and Determination of Hepatic Apoptosis in Rats

ABSTRACT

Aims: The present study was designed to evaluate the hepatoprotective activity of grape seed oil (GSO) on liver lesions induced by carbon tetrachloride (CCl_4) in rats by measurement of caspase 3, 8 and 9 activities in cellular apoptosis, ALT activities, triglyceride, total protein, total cholesterol and liver MDA levels.

Place and Duration of Study: Faculty of Veterinary Medicine, Department of Pathology, Erciyes University, Kayseri, between November 2017 and September2018

Methodology: In this study 40 male Wistar albino rats were divided into four groups including 10 animals in each. Control group administered with 0.9% NaCl. The second group was administered with 4 mL/kg GSO for twelve weeks. Third group were given CCl_4 (0.2 mL/kg) twice for 8-weeks. Fourth group was administered with 4 mL/kg GSO, for 12 weeks and also given CCl_4 (0.2 mL/kg) twice for 8 weeks, starting from the 5th week.

Results: Histopathological examination of CCl_4 group showed intense macro and micro vesicular steatosis in hepatocytes, necrosis, and lymphocytes rich mononuclear cell infiltration in portal area and mild portal fibrosis in the parenchyma of the incomplete formation of lobulation in the parenchyma. The grape seed oil application applications have partially normalized the altered histological changes and the activity of caspase -3, -8 and -9. Administration of GSO led to a decline in the activities of ALT and MDA levels while this treatment elevated serum triglycerides levels which are not significantly important.

Conclusion: The results indicate that the antioxidant properties of GSO have not ameliorative effect in either the histopathological lesions or biochemical parameters against CCl_4-induced hepatotoxicity in rats. Also, it has been concluded that duration-dependent further research results are needed to determine the effects of grape seed oil in high doses which can give the best results without side effects.

Keywords: Histopathology, immunhistochemistry, carbon tetrachloride, grape seed oil, rat.

1. INTRODUCTION

Liver is an organ that is most exposed to toxic substances due to anatomic localization and important functions and can be damaged by many factors [1]. Carbon tetrachloride shows its effects at the level of biochemical and cell organelles in acute and chronic intoxication [2, 3]. Oxidative stress and subsequent free radicals are known to cause damage in tissues. Free radical derivatives result from the formation of oxidative stress and produce lipid peroxidation by acting on unsaturated fatty acids in the cell membrane [4, 5, 6]. It is also suggested that Kupffer cells may be implicated in the pathogenesis of liver damage by the action of proinflammatory mediators (nitric oxide, etc.) released from activated Kupffer cells [7].

The oxidative stress has been the focus of research in recent years. Experimental animal model studies that use extracts and oils of plants with an antioxidant content prevents lipid peroxidation, have become recently popular for the determination of the protective effects of toxic chemicals against liver damage because they are cheap and easily accessible and have nontoxic and low side effects [8]. It has been reported that grape seed oil has free radical scavenging and antioxidant effect and may have protective effect on CCl_4-induced liver injury [9, 10, 11, 12]. This study aimed to determine the effects of GSO, which is known to have various biological activities on CCl_4 induced hepatic damage, by assaying serum ALT (alanine amino transferase) activity, triglyceride, total protein, total cholesterol, liver MDA and total antioxidant capacity levels as well as the immunohistochemistry analyses of apoptosis by caspase 3, -8, and -9 activities of liver tissues in rats.

2. MATERIAL AND METHODS

2.1. Materials

Grape seed oil (GSO) used in the study is commercially available from BUKAS and its components are shown in Table 1.

Table 1. Fatty acid composition of the grape seed oil used in the experiment.

Saturated Fatty Acid	**Percentage**
Myristic acid	0.05
Palmitic Acid	8.56
Palmitoleic Acid	0.18
Margaric Acid	0.07
Stearic Acid	4.41
Unsaturated Fatty Acid	**Percentage**
Oleic Acid (Omega 9)	22.00
Linoleic Acid (Omega 6)	64.47
Linolenic Acid (Omega 3)	0.32
Arachidic Acid	0.15
Eicosenoic Acid	0.15
Total	**100**

2.2. Animals

Experiments were performed using 200–250 g weighing, 40 adult male Wistar albino rats. The experiments were carried out in accordance with the Guidelines for Animal Experimentation approved by Erciyes University, Experimental Animal Ethics Committee (permit no: 17/054), and the experimental procedures were performed in Erciyes University Experimental Research and Application Center, Kayseri, Turkey. The animals were kept in a special room at a constant temperature 22℃ ± 2℃ and humidity (50% ±5%) with 12-h light/dark cycles and had free access to diet and tap water.

2.2. Experimental protocol

They were divided into 4 groups, each containing 10 animals. The first group was identified as control and 0.9% NaCl (1 ml/kg/live weight). The second group was administered with 4 mL/kg/live weight GSO for twelve weeks each day. Third group were given CCl_4 (0.2 mL/kg)

twice for 8-weeks. Fourth group was administered with 4 mL/kg GSO, for 12 weeks and also given CCl_4 (0.2 mL/kg 1:1 ratio of corn oil) twice for 8 weeks, starting from the 5th week.

2.3. Collection and processing of samples

The rats were anesthetized with intramuscular 80 mg/kg ketamine (alfamine, 100 mg/ml, Ata-Fen) and 12 mg/kg xylazine (alfazyne, 20 mg/ml, Ata-Fen) injection [13] 24 hours after the last CCl_4 application. After the chest cavities were opened, intracardiac blood samples were taken in anticoagulant and coagulant tubes and necropsies were performed. Blood samples were centrifuged at 3000 rpm for 10 min and then serum and plasma were separated and stored at -20℃ until analysis were done. All tissue samples were placed in a 10% buffered neutral formalin solution for light microscopic examination [14]. A portion of the liver tissue was stored at -80℃ until the day of study to determine MDA. Serum ALT activity, triglyceride, total protein and cholesterol levels were determined by using commercial kits (Roche Cobas Kit-Switzerland) with auto-analyzer (Roche Cobas 8000) in Gulser- Dr. Mustafa Gundogdu Central Laboratory, Erciyes University. Liver tissue MDA (Cayman, USA, cat no. 10009055) levels were determined with ELISA (CayQuant Bio-Tek, ELx50, USA) by using commercial kits.

Following fixation in neutral formalin solution (10%), liver tissue specimens were thoroughly rinsed overnight, under tap water. Then, all tissue samples were dehydrated in graded alcohol and cleared in xylene, and embedded in paraffin wax and sectioned (thickness, 5 µm), for histopathological evaluation. After staining with hematoxylin and eosin [18] sections were examined with light microscope. To demonstrate caspase activity in tissues, the Avidin Biotin Peroxidase Complex (ABC) technique was performed according to the standard procedure provided in the commercial kit (Zymed, Histostain Plus Kit, California, USA). Anti-caspase-3 (active) (Novus NB100-56113) (dilution ratio 1/2000), anti-caspase-8 (Abcam ab25901) (dilution ratio 1/100) and anti-caspase-9 (Abcam ab25758) (dilution ratio 1/100) were used as primary antibodies. As a negative control PBS was applied to liver tissues and as a positive control, primary antibodies were applied to the control tissues recommended by the primary antibody manufacturers.

All sections were semi quantitatively evaluated for hepatocyte steatosis, inflammation, necrosis and fibrosis using ten different places in each section for the aforementioned parameters by two pathologists and the mean percentile values within the groups were calculated. The values obtained in each group were evaluated statistically and the importances between the groups were recorded. The significance of the difference between the experimental and control groups for liver tissue damage score were made by Kruskal-Wallis test. Statistical analyses were carried out using SPSS 20.

3. RESULTS AND DISCUSSION

In both the control (group 1) and GSO (group 2) groups, no clinical signs were observed, whereas in the CCl_4 and CCl_4+GSO groups, the most remarkable signs were exhaustion, dysorexia, weakness and hypersalivation.

The histopathological examination of the rats revealed normal liver tissue samples in groups 1 (Figure 1A) and 2 (Figure 1B). The histopathological examination of liver tissues in carbon tetrachloride group (group 3), revealed dense macro and micro-vescular fat vacuoles in the hepatocytes (Figure 1C). Especially close to the portal area, lymphocyte-rich mononuclear cell infiltrations and Kupffer cells increased in number and focal hemorrhage areas were seen. There was also increased fibrous connective tissue between the lipid vacuoles (Figure 1D). The histopathological examination of the liver of rats in GSO+CCl_4 group (group 4) the appearance of lesions were similar with group 3 (Figure 1E, 1F).

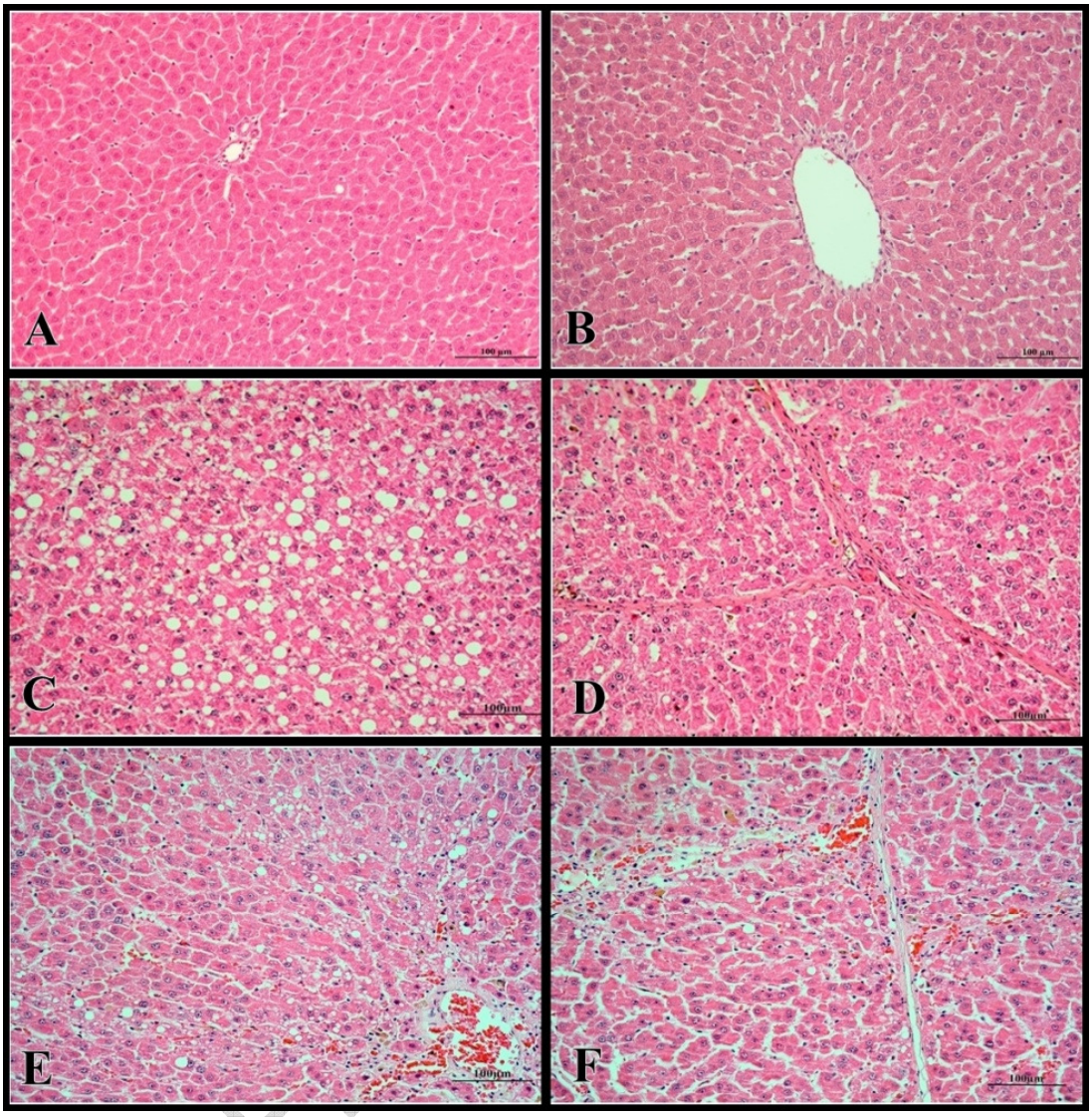

Fig. 1. Histological analysis of the livers in carbon tetrachloride-induced chronic hepatotoxicity; Normal appearance of the livers of the group 1 (A) and group 2 (B) groups. The appearance of micro-macro vesicular fat vacuoles in all parenchyma and there was also increased fibrous connective tissue between the lipid vacuoles in group 3 (C, D) and group 4 (E, F), Liver, HxE.

The staining of caspase 8 in tissue sections of liver was negative in groups 1 and 2. However, in few hepatocytes exposed to normal apoptosis, caspase 3 and caspase 9 were found to be positive (Figures 2). The examined liver sections of group 3, caspase 3, caspase 8 and caspase 9 cytoplasmic immunopositive cells were detected particularly in the periphery of hepatocytes with lipid vacuoles (Figure 3A, 3B, 3C). Immunohistochemical examination of group 4, the severity of positivity in caspase 3, caspase 8 and caspase 9 was similar to CCl_4 group in hepatocytes in the periphery of sentriacinar veins (Figure 3D, 3E, 3F).

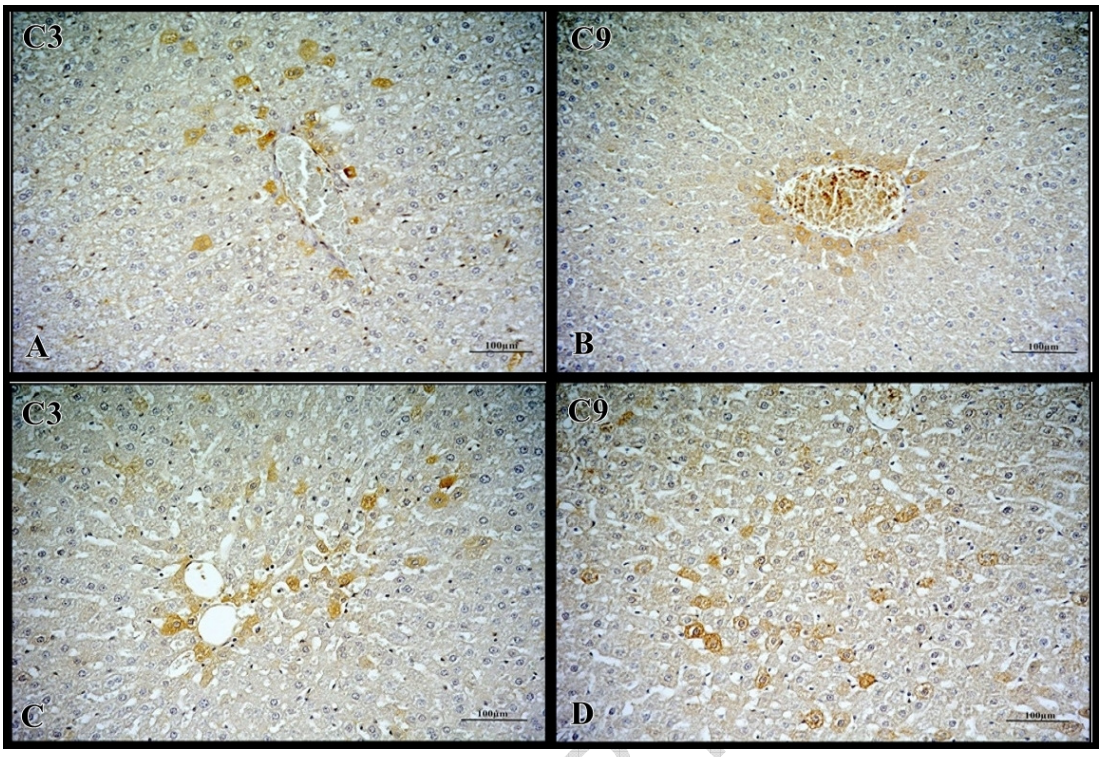

Fig. 2. Hepatic active caspase 3 (C3) and 9 (C9) expression. Hepatic caspase 3 and caspase 9 immunstaining of group 1 (A, B) and group 2 (C, D). ABC-P, Magnificaiton x100.

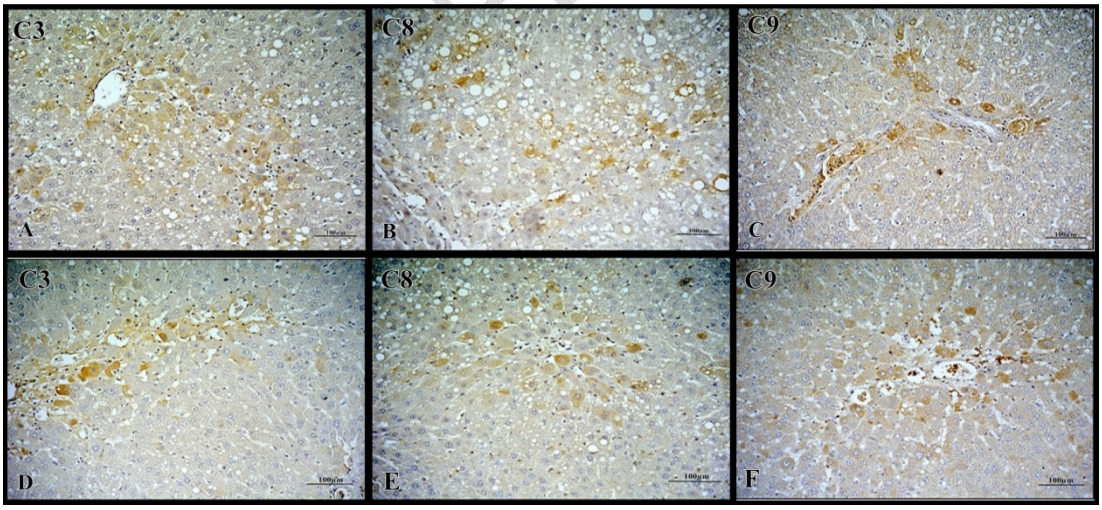

Fig. 3. Hepatic active caspase 3 (C3), caspase 8 (C8) and caspase 9 (C9) expression. Caspase 3, caspase 8 and caspase 9 immunoreactivity in the livers of CCl_4-intoxicated rats in group 3 (A, B, C) and group 4 (D, E, F) showed brown stained cytoplasm. ABC-P, Magnificaiton x100.

In the both of group 1 and 2 liver damage scores were found to be zero. While the difference between the groups 3 and 4 in terms of fibrosis, inflammation, steatosis and necrosis scoring was statistically insignificant (Table 2).

Table 2. Scoring system for hepatic damage in CCl_4 treated groups (n=8; *P* < ,001).

	Control (N=10) Median (%25-%75)	CCl_4 (N=10) Median (%25-%75)	GSO (N=10) Median (%25-%75)	GSO+CCl_4 (N=10) Median (%25-%75)	P
Inflammation	0^{a} (0-0)	$1,5^{b}$ (0,75-2,25)	0^{a} (0-0)	$0,5^{a}$ (0-1)	***P* < .001**
Steatosis	0^{a} (0-0)	2^{b} (2-3)	0^{a} (0-0)	1^{b} (0,75-2)	***P* < .001**
Necrosis	0^{a} (0-0)	0^{a} (0-1)	0^{a} (0-0)	0^{a} (0-1)	***P* > .05**
Fibrosis	0^{a} (0-0)	$0,5^{a}$ (0-1)	0^{a} (0-0)	1^{a} (0-1)	***P* > .05**

[a-b]: the difference between groups in the same line with different letters is statistically significant

At the end of the experiment, no statistically difference in biochemical parameters (serum ALT activity, triglyceride, total protein, cholesterol and MDA levels) were determined between Group 1 and 2 (Table 3). The present study has shown a significant elevation in serum ALT activity, total cholesterol, triglyceride and MDA levels (*P* <.01) with a significant decrease in serum total protein levels (*P* <.01) after CCl_4 administration compared to the control group (Table 3). Serum ALT activities, total cholesterol and MDA levels were affected from GSO administration.

Table 3. Effects of GSO on serum ALT activities, total protein, total cholesterol, triglycerides and MDA levels of rats in control and CCl_4 treated groups.

	CONTROL (N=10)	CCl_4 (N=10)	GSO (N=10)	GSO+CCl_4 (N=10)	P
ALT(U/L)	$64,0^{b}$ (75,0-100,5)	$105,0^{a}$ (82,0-135,5)	$72,0^{b}$ (62,75-76,25)	$93,0^{a}$ (82,75-109,5)	***P* < .01**
Total Protein(g/dL)	$6,6^{b}$ (6,5-6,8)	$5,9^{a}$ (5,7-6,1)	$6,3^{b}$ (6,0-6,5)	$6,1^{b}$ (5,9-6,3)	***P* < .01**
Total cholesterol (mg/dL)	$59,5^{b}$ (55,7-67,0)	$81,0^{a}$ (71,0-82,0)	$56,5^{b}$ (55,0-64,0)	$74,0^{a}$ (65,25-77,5)	***P* < .01**
Triglycerides (mg/dL)	$72,5^{b}$ (60,25-86,5)	$171,5^{a}$ (120,0-196,0)	$101,5^{b}$ (95,25-114,2)	$110,5^{b}$ (99,5-138,75)	***P* < .01**
MDA (µmoL/mg protein)	$19,62^{b}$ (18,1-21,37)	$24,78^{a}$ (22,64-28,38)	$20,34^{b}$ (17,94-20,82)	$21,78^{b}$ (20,34-22,38)	***P* < .01**

(n:10, GSO: Grape seed oil, [a-b]: the difference between groups in the same line with different letters is statistically significant)

Due to the fact that many drugs of plant origin are cheap and easily accessible, they have been popularized in the 20th century in order to determine the antioxidant and liver protective effects against liver damage caused by different chemical toxins [8]. Liver damage caused by chronic carbon tetrachloride administration characterized by hepatitis, fibrosis and cirrhosis in the liver of rats.

Carbon tetrachloride transformed to trichloromethyl (CCl_3) and trichloromethyl peroxyl (CCl_3O_2) free radical metabolites by cytochrome P450 enzyme system in liver microsomes. These toxic metabolites are thougt to interact with membrane lipids which induce lipid peroxidation [4, 5].

In chronic injury induced by CCl_4 generate free radicals and there is an increase in the deposition of extracellular matrix resulting in severe fibrosis and subsequent lead to liver cirrhosis. The rats were administered CCl_4 three times a week for eight weeks [15], once a week for ten weeks [16], twice a week for twelve weeks [17], and twice a week for thirteen weeks [18], have been shown to cause fibrosis, severe necrosis, and pseudolobulation formation in the hepatic tissue, especially in the portal area, with inflammatory cell infiltrations with macro-microvesicular fat vacuoles in hepatocytes. In the present study, in accordance with the findings of the researchers [15, 16, 17, 18, 19], the application of CCl_4 at a dose of 0.2 mL/kg for eight weeks resulted in the formation of fibrosis in the liver parenchyma of the rats with a severe severity of inflammation in the liver parenchyma. The histopathological changes are caused by the toxic metabolites [5, 20] of CCl_4 which initiate lipid peroxidation by disrupting the passage of ions from the cell membrane and [21] also by increasing oxidative stress causing mitochondrial damage in hepatocytes.The histopathological changes; the passing of ions from the cell members by the toxic metabolites of CCl4 , initiating lipid peroxidation, the activation of membrane enzymes and intracellular signal transduction [21], as well as increasing oxidative stress causing mitochondrial damage in hepatocytes. Histopathological changes; Lipid peroxidation of the toxic metabolites of CCl_4 [5, 20] by breaking the passage of ions from the cell membrane [21] also caused by increased oxidative stress mitochondrial damage in hepatocytes.

Grape seed extract and oil contain a large number of polyphenols, including procyanidins and proanthocyanidins and they are highly potent free radical scavengers [22]. Grape seed extract shows potent antioxidant, anti-inflammatory and anticarcinogenic activities as well as inhibition of apoptosis which attributed to its high content of polyphenols [23, 24]. Procyanidins are natural antioxidants and have biological and therapeutic effects against free oxygen radicals and oxidative stress by inhibiting free oxygen radicals accordance with their concentrations. Procyanidins are natural antioxidants and have biological, pharmacological and therapeutic effects against free oxygen radicals, inhibiting free oxygen radicals depending on concentration [25]. Proanthocyanidins are the metabolites of natural plant and are commonly found in fruits, vegetables, nuts, flowers, wine, black and green tea, and etc. [26]. Due to their strong anti-oxidant activity, several studies have been conducted to evaluate its anti-carcinogenic, antiinflammatory, antimicrobial, antiallergic, antifungal, antiarthritic, antiviral, immunostimulant, cardioprotective and vasodialatator effects on different conditions [25, 26].

In studies in which grape seed oil was given to improve chronic liver damage caused by CCl_4 [10, 11, 12] and other toxic substances [27, 28, 29, 30], histopathological changes were shown to be decreased. The results of previously published studies [10, 11] with carbon tetrachloride in rats suggested a recovery with the treatment of grape seed extract [31] while Atasever et al. showed that grape seed extract had no ameliorative effect on liver damage.

There are limited numbers of studies using grape seed oil on carbontetrachloride-induced chronic liver damage. Maheswari and Rao [12] reported that grape seed oil decreased the apperance of fatty degeneration, necrosis and fibrosis induced by CCl_4 in rats. In the present study, grape seed oil has been shown to slightly reduce the number of fat vacuoles and partially reduce necrosis areas and prevent the fibrous tissue formation in the liver. Many studies [27, 28] have reported that grape seed oil causes histological improvement in liver lesions caused by other toxic substances. In studies conducted with grape seed oil against various hepatotoxins, the hepatoprotective effect of grape seed oil is thought to be due to

antioxidant and free radical scavenging components. However, further researches should be conducted to provide a better understanding of the subject.

Because some enzymes are specific to the tissues, their increase in blood levels is used for the clinical diagnosis of the diseases characterized by degeneration and necrosis in some tissues such as liver, kidney, heart and skeletal muscle. An increased enzyme activity of ALT is related to hepatic parenchymal damage. Alanine aminotransferase is an enzyme that increases in blood levels in hepatic diseases [32]. It is well known that agents such as CCl_4 which leads to injury in liver parenchyma, cause an increase in plasma ALT activities [33].

Malondialdehyde is the main product of lipid peroxidation in cell membrane systems. Malondialdehyde as the final product of lipid peroxidation leads to the formation of hydrogen peroxide and reactive oxygen species leading to ozone formation and membrane denaturation and peroxidation [34, 35]. On the other hand, it has been reported that nitric oxide released from Kupffer cells, endothelial cells and hepatocytes is an important mediator in the inflammation and tissue damage caused by CCl_4 [7, 36].

Maheswari and Rao [12] reported that grape seed oil normalized the serum ALT activities and liver MDA levels induced by CCl4. In the present study, grape seed oil did not cause any changes in ALT activity. However, MDA levels were significantly reduced which is similar with the results of Maheswari [12].

There are biochemical data in studies [10, 27, 28, 29, 31, 30] using grape seed oil or extract for the treatment of toxicity with different chemicals other than CCl_4 in liver.

Al-Attar [27] showed that triglyceride, cholesterol levels and ALT enzyme activities increased significantly in diazinone treated animals, while serum total protein levels were significantly decreased; these values were close to the control group values in the GSO group; Khalifa et al. [28] reported that GSO decreased serum ALT and MDA levels in rats with Chlorpyrifos intoxicaiton; Atasever and Yaman Gram have reported that [31] grape seed oil decreased serum total protein, albumin and globulin levels, while ALT activities increased in CCl4-induced liver injury in rats; Al-Ashmawy et al. [29] reported that MDA levels decreased with grape seed extracts on ethanol toxication; Shin and Moon [30] have reported that grape seed reduced, liver MDA, serum albumin and total protein levels in dimethylnitrozamine intoxication, Li et al. [10] reported that grape seed extract decreases serum triglycerides and MDA levels against CCl_4 toxicitation in rats with.

Hepatocyte apoptosis can occur in liver damage such as drug intoxications, alcohol and viral infections [37, 38, 39]. Carbon tetrachloride destroys the mitochondrial phospholipid bilayer in hepatocytes and induces caspase 3 dependent apoptosis. In vitro and in vivo studies have shown that hepatocyte apoptosis is determined immunohistochemically with caspase activity in CCl_4 induced liver damage [40, 41, 42].

In the present study, the increase in caspase 3, 8 and 9 activities in the CCl4 administered groups was found similar to the findings of the earlier studies [40, 41, 42]. The application of GSO partially reduced the activities of caspase 3, 8 and 9, and thus hepatocyte apoptosis. In this study grape seed oil reduced the activity of caspase-3, -8 and caspase-9, which suggested that grape seed oil could protect the liver tissue.

4. CONCLUSION

As a result, the ameliorative effect of 4 mL/kg dose of GSO on the liver injury was determined by biochemical parameters. However, this amelioration did not reflect on histological damage to the liver tissue of rats. It is also concluded that new investigations is

needed to be performed to determine the ameliorative effects of grape seed oil on tissues using the doses to give the best results without any side effects.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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